

## A PROTOCOL FOR *IN VITRO* SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS OF *ANDROGRAPHIS PANICULATA* WALL. EX NEES

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*Andrographis paniculata* Wall. Ex Nees is a small branched annual medicinal herb. *In vitro* study was conducted for somatic embryogenesis through *in vitro* leaf explant on MS medium containing different doses of 2,4-D and BA. 4.44  $\mu$ M BA with the combination of 0.45  $\mu$ M 2,4-D produced maximum somatic embryos with in 60, i.e. 30+30 (intermittent one mid transfer on the same fresh medium) days after inoculation of trimmed leaf explants. Different strength of MS medium (1.0 $\times$ , 0.75 $\times$ , 0.50 $\times$  and 0.25 $\times$ ) tested on somatic embryos for the maturation of embryos. 30 days after the inoculation of somatic embryos, 0.50  $\times$  gave 96% mature embryos. After that these mature embryos developed into plants on MS medium containing 5  $\mu$ M GA3 and 10  $\mu$ M BA in 30 days after the inoculation of mature embryos. The somatic embryogenesis raised plantlets acclimatized and successfully transferred to the field and successfully survived.

**Keywords:** Embryogenic callus; Embryo germination; Embryo maturation; Plantlet recovery

The *in vitro* explant, i.e. a vegetative propagule or mother tissue can regenerate into an adventitious plant through two alternative mechanisms, namely organogenesis and somatic embryogenesis. In the first case, adventitious shoots and roots are sequentially regenerated and characterized by the organic (vascular) connections between *de novo* adventitious shoots and the mother tissue (Terzi and Lo Schiavo, 1990). On the other hand, somatic embryogenesis involves orderly processes of multiplication, differentiation and maturation of haploid or diploid somatic cells into discrete bipolar structures, i.e. somatic embryos without organic connections with mother tissue, sharing structural and functional similarity with zygotic embryos (Williams and Maheswaran 1986, Emons 199, Raemakers *et al.* 1995). He *et al.* (1990) have reported the occurrence of both organogenesis and somatic embryogenesis in the same explant, which originates from particular tissue layers or cells within explants (Osternack *et al.* 1999). However, somatic embryogenesis is nowadays best known as a pathway to induce *in vitro* regeneration of disease (virus) free plantlets of

genetic uniformity from a proven superior mother plant and provide an opportunity for genetic transformation and production of genetically modified plants.

The present communication describes a standardized protocol for *in vitro* somatic embryogenesis and recovery of plantlets in *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae). The species has been used in traditional systems of medicine to treat much number of ailments, including the common cold, fever, diarrhea, liver diseases, and inflammation (Anonymous 2002). It is a herb that originated from India and widely distributed in southern China. Recently, its active diterpene lactone andrographolides have been clinically demonstrated to exhibit anti HIV activity (Basak *et al.* 1999, Calabrese *et al.* 2000) and cardio-protecting activity (Woo *et al.* 2008) as well as inhibit various oncogenic growths, e.g. prostate cancer (Chun *et al.* 2010) and lymphoma tumors (Yang *et al.* 2010). However, the poor germination of *A. paniculata* seeds (Rawat and Vshishtha 2011; Kumar *et al.* 2011, Bhattacharya *et al.* 2012) poses a problem for its large scale cultivation

and encourages for alternative procedures like somatic embryogenesis for steadily supply of propagules and planting material.

## MATERIALS AND METHODS

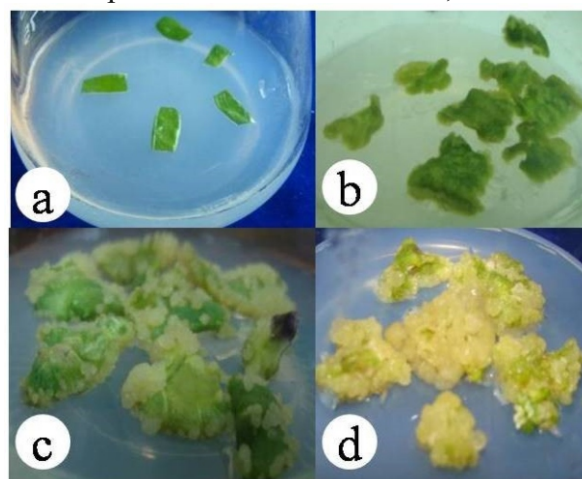
Three sequential experiments were conducted for *in vitro* regeneration of callus and somatic embryogenesis. The dark green leaf explants obtained from *in vitro* raised shoots of *Andrographis paniculata* on MS medium supplemented with 5  $\mu\text{M}$  BA were used for callus induction in Experiment 1. The leaf explants were trimmed at base and tips and cut at midribs before inoculation abaxially on the culture medium (Fig. 1a). The first and third experiments were conducted in factorial randomized design; whereas the second experiment was carried out in a simple randomized design. Four doses each of 2,4-D (0, 0.45, 2.26 and 4.52  $\mu\text{M}$ ) and BA (0, 4.44, 6.66 and 8.88  $\mu\text{M}$ ) and their all possible interactions on callus induction from leaf explants in Experiment 1, MS medium strengths (1.0 $\times$ , 0.75 $\times$ , 0.50 $\times$  and 0.25 $\times$ ) on somatic embryo maturation in Experiment 2, and four doses each of GA<sub>3</sub> (0, 2.5, 5.0 and 10.0  $\mu\text{M}$ ) and BA (0, 5, 10 and 20  $\mu\text{M}$ ) and their all possible interactions on germination and elongation of matured somatic embryos in Experiment 3 were tested. Samplings were done at 15, 20 and 30 days after inoculation in experiment 1 and 30 days after inoculation in experiment 2 and 3. The data were also recorded for rough embryogenic green callus and smooth non-embryogenic yellow callus at 30 days after inoculation in experiments 1 and 2. Further, the obtained embryogenic callus was uniformly kept for 30 days on MS medium supplemented with standardized combined doses of BA (4.44  $\mu\text{M}$ ) and 2,4-D (2.26  $\mu\text{M}$ ) of experiment 1 before being used as explants for experiment 2. There were three replicates each of five explants comprising leaf segments for experiment 1 and matured somatic embryos for Experiment 3 and five replicates each of five

explants i.e. embryogenic callus for experiment 2.

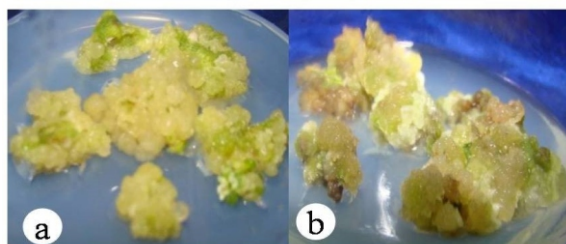
All cultures were maintained at  $25 \pm 2^\circ\text{C}$  under a light intensity of  $45 \mu\text{Em}^{-2}\text{s}^{-1}$  with a 16 h photoperiod. The full-strength MS medium (unless otherwise mentioned) supplemented with 30 g/L sucrose and made semi-solid with 7.5 g/L agar was uniformly used in all experiments. The pH of the medium was adjusted to 5.7 to 5.8 before autoclaving for 20 min at  $121^\circ\text{C}$  and  $1.46 \times 10^5$  Pa. Data were subjected to ANOVA analysis following F test at  $P < 0.05$ . If the F test found significant, Tukey's LSD values at  $P = 0.05$  were computed for comparing parameter values at various time intervals.

## RESULTS

**Callus induction:** Leaf explants inoculated in Experiment 1 exhibited swelling at 15 days (Fig. 1a), callus formation in 20 days (Fig. 1c) and differentiating callus in 30 days (Fig. 1d) after inoculation. 2,4-D/BA doses alone and their in combinations significantly influenced various parameters recorded at 15, 20 and 30

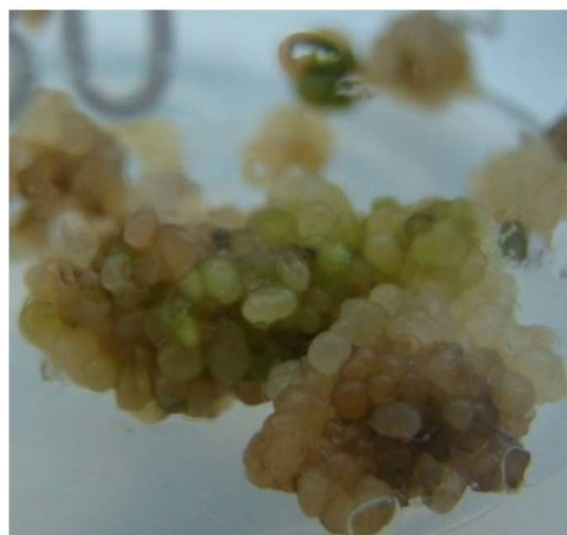


**Figure 1.** The *in vitro* rough embryogenic callus induction from leaf segment of *Andrographis paniculata* on MS medium supplemented with 0.45  $\mu\text{M}$  2,4-D and 4.44  $\mu\text{M}$  BA. (a) leaf explants at 0 day, (b) leaf explants swelling at 15 days, (c) callus formation at 20 days and (d) embryogenic callus at 30 days after inoculation.



**Figure 2.** The *in vitro* callus induction from leaf segment of *Andrographis paniculata* at 30 days after inoculation. (a) Embryogenic callus on MS medium supplemented with 0.45  $\mu\text{M}$  2,4-D and 4.44  $\mu\text{M}$  BA and (b) Non-embryogenic callus on MS medium supplemented with 2.26-4.52  $\mu\text{M}$  2,4-D and 6.66-8.88  $\mu\text{M}$  BA.

days after inoculation (Tables 1-3). Leaf swelling at 15 days and callus formation at 20 days were significantly maximum in 0.45- 4.52  $\mu\text{M}$  2,4-D, 4.44-6.66  $\mu\text{M}$  BA and their interactions (Tab. 1-2). Total callus 30 days was significantly highest at 4.52  $\mu\text{M}$  2,4-D, 4.44-8.88  $\mu\text{M}$  BA and all interactions comprising 0.45-4.52  $\mu\text{M}$  2,4-D and 4.44-8.88  $\mu\text{M}$  BA. However, the rough embryogenic callus (Fig. 2a) was significantly highest at 0.45  $\mu\text{M}$  2,4-D, 4.44  $\mu\text{M}$  BA and interactions comprising 0.45-4.52  $\mu\text{M}$  2,4-D with 4.44  $\mu\text{M}$  BA and 0.45  $\mu\text{M}$  2,4-D with 6.66-8.88  $\mu\text{M}$  BA and the smooth non-embryogenic callus (Fig. 2b) in 4.52  $\mu\text{M}$  2,4-D, 6.66-8.88  $\mu\text{M}$  BA and interactions comprising 2.26-4.52  $\mu\text{M}$  2,4-D and 6.66-8.88  $\mu\text{M}$  BA (Tab. 3). Leaf explants maintained on



**Figure 3.** The *in vitro* embryogenic callus from leaf segment of *Andrographis paniculata* obtained on MS medium supplemented with 0.45  $\mu\text{M}$  2,4-D and 4.44  $\mu\text{M}$  BA at 30 days after inoculation used for maturation of somatic embryos in Experiment 3.

hormone-free culture medium did not exhibit swelling or callus induction at all stages of sampling (Tab. 1-3). MS medium supplemented with 0.45  $\mu\text{M}$  2,4-D and 4.44  $\mu\text{M}$  BA emerged to be the most economical and judicious for bulk production of rough embryogenic callus (Fig. 3), which was subsequently used for Experiment 2 dealing with the maturation of somatic embryos.

**Table 1.** Effect of BA doses (B), 2,4-D doses (D) and their interactions on leaf explant swelling (%) at 15 days after inoculation. Values expressed in the parenthesis are arc sine transformed. Data are mean of three replicates.

2,4-D ( $\mu\text{M}$ )	BA ( $\mu\text{M}$ )				Mean	Variable	LSD <sub>(0.05)</sub>
	0	4.44	6.66	8.88			
0	0 (5.00)	0 (5.00)	0 (5.00)	0 (5.00)	0 (5.00)	B	6.8
0.45	0 (5.00)	100.00 (85.00)	100.00 (85.00)	80.00 (66.40)	70.00 (60.35)	D	6.8
2.26	46.67 (43.08)	100.00 (85.00)	100.00 (85.00)	86.67 (70.63)	83.33 (70.93)	B*D	13.6
4.52	46.67 (43.08)	100.00 (85.00)	93.33 (77.81)	93.33 (77.81)	83.33 (70.93)		
Mean	23.33 (24.04)	75.00 (65.00)	73.33 (63.20)	65.00 (54.96)			

**Table 2.** Effect of BA doses (B), 2,4-D doses (D) and their interactions on leaf callus induction (%) at 20 days after inoculation. Values expressed in the parenthesis are arc sine transformed. Data are mean of three replicates.

2,4-D (μM)	BA (μM)				Mean	Variable	LSD <sub>(0.05)</sub>
	0	4.44	6.66	8.88			
0	0	0	0	0	0	B	7.2
	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	D	7.2
0.45	0	100.00	100.00	80.00	70.00	B*D	14.4
	(5.00)	(85.00)	(85.00)	(66.40)	(60.35)		
2.26	0	100.00	100.00	86.67	71.67		
	(5.00)	(85.00)	(85.00)	(70.63)	(61.41)		
4.52	20.00	100.00	93.33	93.33	76.67		
	(21.93)	(85.00)	(77.81)	(77.81)	(65.64)		
Mean	5.00	75.00	73.33	65.00			
	(9.23)	(65.00)	(63.20)	(54.96)			

**Table 3.** Effect of BA doses (B), 2,4-D doses (D) and their interactions on induction of different type leaf callus at 30 days after inoculation. Values expressed in the parenthesis are arc sine transformed. Data are mean of three replicates

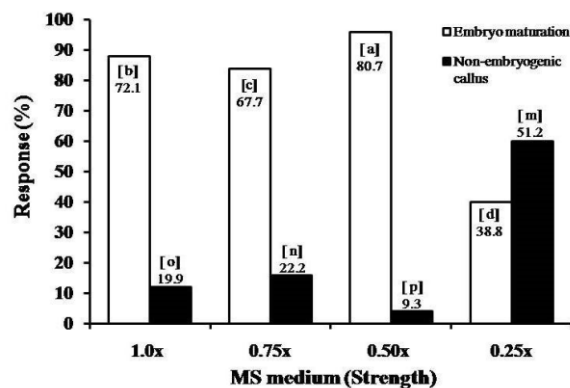
2,4-D (μM)	Callus (%)														
	Total					Embryogenic rough					Non-embryogenic smooth				
	BA (μM)					BA (μM)					BA (μM)				
	0	4.44	6.66	8.88	Mean	0	4.44	6.66	8.88	Mean	0	4.44	6.66	8.88	Mean
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)
0.45	0	100	100	100	75.00	0	100	100	100	75.00	0	0	0	0	0
	(5.00)	(85.00)	(85.00)	(85.00)	(65.00)	(5.00)	(85.00)	(85.00)	(85.00)	(65.00)	(5.00)	(5.00)	(5.00)	(5.00)	(5.00)
2.26	6.67	100	100	100	76.67	0	100	0	0	25.00	6.67	0	100	100	51.67
	(12.21)	(85.00)	(85.00)	(85.00)	(66.81)	(5.00)	(85.00)	(5.00)	(5.00)	(25.00)	(12.21)	(5.00)	(85.00)	(85.00)	(46.81)
4.52	33.33	100	100	100	83.33	0	100	0	0	25.00	33.33	0	100	100	58.33
	(35.01)	(85.00)	(85.00)	(85.00)	(72.50)	(5.00)	(85.00)	(5.00)	(5.00)	(25.00)	(35.01)	(5.00)	(85.00)	(85.00)	(52.50)
Mean	10	75.00	75.00	75.00		0	75.00	25.00	25.00		10	0	50	50	
	(14.31)	(65.00)	(65.00)	(65.00)		(5.00)	(65.00)	(25.00)	(25.00)		(14.31)	(5.00)	(45.00)	(45.00)	

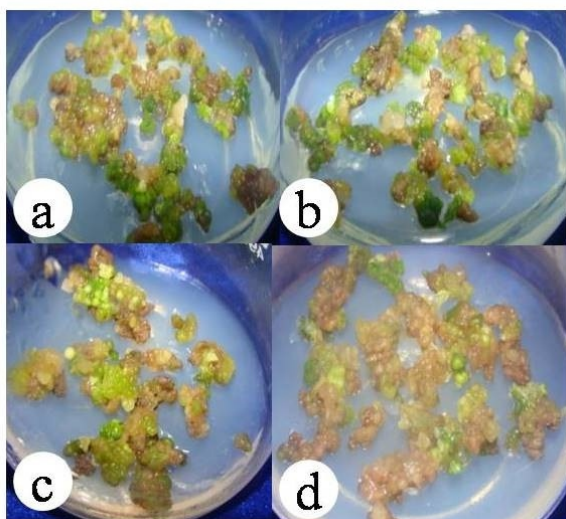
Variable	LSD <sub>(0.05)</sub>		
	Total	Rough	Smooth
B	3.4	*	3.4
D	3.4	*	3.4
B*D	6.8	*	6.8

\*There was no residual value for error term in ANOVA, hence CD value being zero, which means each value arising from both treatments and their all possible interactions was distinguished significantly from other values

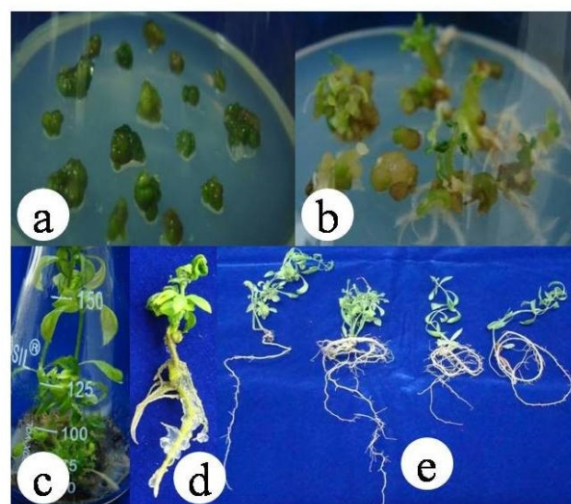
**Somatic embryo maturation:** MS medium strengths significantly influenced somatic embryo maturation at 30 days after inoculation of embryogenic callus (Fig. 4-5). Of the four strengths, 0.50x MS medium was found to be significantly optimum for the parameter, producing 96% mature somatic embryos (Fig. 4). The significantly lowest somatic embryo maturation was recorded on 0.25× MS. The embryo maturation was 2.4 times higher in 0.50× MS medium than 0.25× MS medium. Further, 0.25× MS medium also produced the highest amount of non-embryogenic callus.



**Figure 4.** The *in vitro* maturation of somatic embryos of *Andrographis paniculata* on various strengths of hormone free MS medium at 30 days after inoculation. Two series of alphabets are used for somatic embryo maturation (a, b, c, d) and non-embryogenic callus (m, n, o, p). The histograms bearing different alphabets represent statistically different values in their series. The numerical values on histograms are arc sine transformations of the corresponding data.



**Figure 5.** The *in vitro* maturation of somatic embryos of *Andrographis paniculata* on various strengths of hormone free MS medium at 30 days after inoculation. (a) 1.0× MS, (b) 0.75× MS, (c) 0.5× MS and (d) 0.25× MS.



**Figure 6.** The *in vitro* germination of somatic embryos and recovery of plantlets of *Andrographis paniculata* on MS medium supplemented with 10  $\mu\text{M}$  BA and 5  $\mu\text{M}$  GA<sub>3</sub>. (a) Greening/(b) germination at 30 days after inoculation, (c) bulk germination in flask, (d) a single plantlet from bulk germination and (e) 170-180 days old plantlets from somatic embryos used for hardening.

**Table 4:** Effect of BA doses (B), GA<sub>3</sub> doses (G) and their interaction (B\*G) on shoot and root number at 30 days after inoculation. The data are mean of three replicates

BA ( $\mu\text{M}$ )	GA <sub>3</sub> ( $\mu\text{M}$ )					Variable	LSD <sub>(0.05)</sub>						
	Shoot number SE <sup>-1</sup>						Root number SE <sup>-1</sup>		Shoot number	Root number			
0	2.5	5.0	10.0	Mean	0	2.5	5.0	10.0	Mean				
0	1.00	0.67	0.67	0.33	0.67	0	0	0.67	0.67	0.33			
5.0	0	0	1.33	1.00	0.58	0	0	2.00	0.33	0.58	B	0.92	0.60
10.0	1.33	1.67	3.67	0.67	1.83	1.33	1.00	1.67	1.00	1.25	G	0.92	0.60
20.0	0.67	0	2.33	0	0.75	0	0.33	1.33	0	0.42	B*G	NS	NS
Mean	0.75	0.58	2.00	0.50	0.33	0.33	1.42	0.50					

**Germination of the somatic embryo and plantlet recovery:** BA and GA<sub>3</sub> alone significantly promoted germination of somatic embryos in 30 days after inoculation (Tab. 4, Fig. 6 a, b). MS medium supplemented with 10  $\mu\text{M}$  BA or 5  $\mu\text{M}$  GA<sub>3</sub> produced significantly the highest number of shoots and roots per somatic embryo. 10  $\mu\text{M}$  BA enhanced shoot number by 3.16 and root number by 3.79 times in comparison to that of 5  $\mu\text{M}$  BA. Similarly, 5  $\mu\text{M}$  GA<sub>3</sub> enhanced shoot number by 4 times and root number by 4.30 times in comparison to that 2.5  $\mu\text{M}$  GA<sub>3</sub> or without GA<sub>3</sub>.

## DISCUSSION

Three experiments were sequentially

conducted to induce callus formation and somatic embryogenesis on leaf explants of *A. paniculata*. Callusing primarily depends on the external supply of the phytohormones in the basal media. In the present investigation, the combinations of the hormone supplements (2,4-D and BA) to MS basal medium produced calli. However, 2,4-D alone produced inadequate callus and adequately high callus with the incorporation of BA (Tab. 2). The *in vitro* calli were of two types: the first one was friable rough embryogenic callus on low dose combination of 0.45  $\mu\text{M}$  2,4-D and 4.44  $\mu\text{M}$  BA and second was smooth non-embryogenic callus on a high dose of (2.26- 4.52  $\mu\text{M}$ ) 2,4-D and (6.66 and 8.88  $\mu\text{M}$ ) BA (Tab. 3, Fig. 2).

The above findings are inconsonant with those obtained during somatic embryogenesis from leaf base of wheat by Mahalakshmi *et al.* (2003), who have also reported induction of embryogenic and non-embryogenic calli. They further noted the deleterious effect on regenerative behavior of callus on long exposure to 2,4-D. In our investigation also, the high dose of synthetic auxin (2,4-D) elicits callus on leaf explants, which was less in amount and smooth by appearance. However, incorporation of BA with a low dose of 2,4-D or *vice versa* produces embryogenic callus.

Production of the *in vitro* embryogenic and non-embryogenic calli arising from leaf explants may be interpreted in terms of genomic DNA methylation/demethylation dynamics, which occur during plant growth and development, both *in vivo* and *in vitro* in different cells/tissues of the same organ/whole organism due to environmental and/or ontogenic signals (Ramchandani *et al.* 1999). Genomic DNA demethylation in a specific cell population or organ always precedes the beginning of any differentiation program, including somatic embryogenesis (Valledor *et al.* 2007). Presumably, the highly methylated cells/tissues could have been the progenitor of non-embryogenic callus in the present investigation. The promotion of non-embryogenic callus by 2.26- 4.52  $\mu\text{M}$  2,4-D and 6.66 and 8.88  $\mu\text{M}$  BA further supports the above interpretation as the high doses of both hormones enhance genomic DNA methylation in wheat seedlings Vlasova *et al.* (1995) and sugarbeet cell lines (Causevic *et al.* 2005).

Auxin and cytokinin ratios determine the *de novo* processes of various regenerations, including somatic embryogenesis. It appears that the incorporation of low doses of synthetic auxin (2,4-D) and cytokinin in the present study has induced the cells of leaf explants to produce embryogenic callus by way of generating new methylation patterns and/or promoting demethylation. The higher doses of

both plant regulators could have offset the endogenous balance, resulting in smooth non-embryogenic callus indicative of abnormal growth and differentiation. However, the *in vitro* embryogenic callus of *A. paniculata* requires a combination of both plant growth regulators, e.g. 2,4-D, NAA and BA (Nor Hasnida 2002), 2,4-D/NAA and kinetin/BA (Martin 2004), and 2,4-D and BA (Jha *et al.* 2012). Of these, Martin (2004) could develop a complete protocol for *in vitro* somatic embryogenesis. The findings in the present investigation and those of the previous studies indicate the versatility of the species about response to auxins (2,4-D and NAA) and cytokinins (BA and kinetin) as far as the *in vitro* production of embryogenic callus is concerned.

Transfer of embryogenic callus on a combined low dose of 2,4-D (0.45  $\mu\text{M}$ ) and BA (4.44  $\mu\text{M}$ ) facilitates conversion into somatic embryos at 30 days after inoculation (Fig. 3), which subsequently obtain maturation on reduced strength (0.50 $\times$ ) of MS medium devoid of hormone (Fig. 4). Exogenous supply of auxin up to the globular stage, i.e. initial 1, 2 or 7 days confers cellular competence for the *in vitro* somatic embryogenesis (Kamada and Harada, 1979; Ammlrato 1985; Nomura and Komamine 1985). The further development from the globular stage to the transitory heart stage gets inhibited in the presence of auxin, indicating the requirement of new gene products, which are otherwise inhibited by auxin (Halperin and Wetherell 1964; Borkird *et al.* 1986).

Besides, the *de novo* differentiation and growth from existing structures such as leaf explants represent system (order) change punctuated by temporarily chaos (disorder), which is marked by the development of mild stress conditions. The stress associated with *in vitro* induction of somatic embryogenesis may result in an overall stress response expressed as chromatin reorganization. An extended chromatin

reorganization is believed to cause an 'accidental' release of the embryogenic program, the latter normally being repressed by a chromatin-mediated gene silencing mechanism (Feher 2005). However, the ability of *in vitro* cultures to generate embryos is limited to a group of cells or a discrete zone of embryogenic callus (Quiroz-Figueroa *et al.* 2006). As for the stress response mechanism, Potters *et al.* (2007) have explained that stress-induced morphogenic response in plant cells could be summarized inhibition of cell elongation, localized stimulation of cell division and alteration of the cell differentiation status. These general cell responses are the same independently of the type of stress, which not only promotes dedifferentiation but can also induce somatic embryo formation. The reason that the embryogenic callus has been transferred on hormone-free reduced strength of MS medium for differentiation, growth and maturation of somatic embryos in *A. paniculata*.

In the literature also, the strength reduction of salts in culture medium including the investigated species in the present study or inclusion of retardants (abscisic acid)/osmoticum, polyethylene glycol) favours the process of induction and maturation of somatic embryos in different plant species, e.g. *Picea glauca* (Attree *et al.* 1991; Kong and Yeung 1995), *Manihot esculenta* (Groll *et al.* 2002), *A. paniculata* (Martin 2004) and *Musa* spp. (Srinivas *et al.* 2006).

The mature somatic embryos obtained so were germinated on full strength MS medium supplemented with 10  $\mu$ M BA and 5  $\mu$ M GA<sub>3</sub> (Tab. 4). Cotyledons initially white in color turned green and underwent germination. These plantlets with dark green leaves grew well and formed roots (Fig. 6e). Varshney *et al.* (2009) and Martin (2004) have also obtained the germination of somatic embryos on MS medium rich in BA and GA<sub>3</sub> in *A. paniculata*. Further, the importance of GA<sub>3</sub> in embryo

germination has also been demonstrated in *Crocus cancellatus* (Karamian and Ebrahimzadeh 2001), *Bouteloua gracilis* (Aguado-Santacruz *et al.* 2001), *Santalum album* (Rai and McComb 2002) and *Eleutherococcus sessiliflorus* (Choi *et al.* 2002) and *Gossypium hirsutum* (Kumria *et al.* 2003).

## CONCLUSION

we present a three-step *in vitro* protocol for somatic embryogenesis in *A. paniculata* that includes production of embryogenic callus on MS medium supplemented with 2,4-D (0.45  $\mu$ M) and BA (4.44  $\mu$ M) for 30 days, followed by maturation of somatic embryos on 0.5 $\times$  MS hormone-free medium for 30 days and germination of somatic embryos and recovery of plantlets on MS medium supplemented with GA<sub>3</sub> (10  $\mu$ M) and BA (5  $\mu$ M) for 30-60 days. Thus, the protocol takes about 120 days for the recovery of plantlets via somatic embryogenesis from leaf explants of *A. paniculata*.

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